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REVIEW FOR THE 100TH ANNIVERSARY

Exploring the phytoplasmas, plant pathogenic bacteria

Kensaku Maejima · Kenro Oshima · Shigetou Namba

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Abstract Phytoplasmas are plant pathogenic bacteria associated with devastating damage to over 700 plant species worldwide. It is agriculturally important to identify factors involved in their pathogenicity and to discover effective measures to control phytoplasma diseases. Despite their economic importance, phytoplasmas remain the most poorly characterized plant pathogens, primarily because efforts at in vitro culture, gene delivery, and mutagenesis have been unsuccessful. However, recent molecular studies have revealed unique biological features of phytoplasmas. This review summarizes the history and recent progress in phytoplasma research, focusing on (1) the discovery of phytoplasmas, (2) molecular classification of phytoplasmas, (3) diagnosis of phytoplasma diseases, (4) reductive evolution of the genomes, (5) characteristic features of the plasmids, (6) molecular mechanisms of insect transmissibility, and (7) virulence factors involved in their unique symptoms.

Keywords Diagnosis · Genome · Phytoplasma · Insect transmission · Molecular classification · Virulence factors

Introduction

Phytoplasmas are plant pathogenic bacteria in the class Mollicutes and are formally called mycoplasma-like organisms (MLOs) (Doi et al. 1967). They are transmitted by insect vectors (leafhoppers, planthoppers, and psyllids) and infect hundreds of plant species worldwide, including many economically important crops, fruit trees, and ornamental plants (Hogenhout et al. 2008; Oshima et al. 2013). Infected plants show a wide range of symptoms including stunting, yellowing, witches' broom (development of numerous tiny shoot branches with small leaves), phyllody (formation of leaf-like tissues instead of flowers), virescence (greening of floral organs), proliferation (growth of shoots from floral organs), purple top (reddening of leaves and stems), and phloem necrosis (Fig. 1). Phytoplasma infection is often fatal and causes devastating damage to global agricultural production. Due not only to difficulties in culturing phytoplasmas in vitro and in manipulating them genetically but also to their recalcitrant biological properties such as restriction to phloem cells, nontransmissibility by manual inoculation, and high vector specificity, these organisms are one of the most poorly characterized groups of plant pathogens. However, recent molecular studies have revealed interesting features of phytoplasmas. This review summarizes the history and recent progress in phytoplasma research, especially in Japan.

Phytoplasmal diseases and discovery of the pathogen

Phytoplasmal diseases have been observed globally. In Japan, mulberry dwarf disease has caused severe damage to mulberry plants, the sole source of food for silkworms, since the Tokugawa period (1603–1868) (Okuda 1972). In 1890, Shirai (the first president of the Phytopathological Society of Japan and the first professor of plant pathology at the University of Tokyo) reported that this disease was transmissible by grafting, although the causal agent remained unknown (Shirai 1890). Since the early twentieth

K. Maejima · K. Oshima · S. Namba (✉)
Department of Agricultural and Environmental Biology, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo 113-8657, Japan
e-mail: anamba@mail.ecc.u-tokyo.ac.jp

Fig. 1 Unique symptoms caused by phytoplasma infection. **a** Phyllody symptoms on hydrangea induced by infection with '*Candidatus* Phytoplasma japonicum' JHP phytoplasma (*right*). **b** Witches' broom symptoms on *Ziziphus jujuba* induced by infection with '*Ca*. P. ziziphi' (*left*). **c** Witches' broom symptoms on *Paulownia tomentosa* induced by infection with '*Ca*. P. asteris' (*left*). Photo courtesy of Dr. Norio Nishimura (Koibuchi College of Agriculture and Nutrition)

century, other phytoplasmal diseases, including paulownia witches' broom disease (Kawakami 1902) and rice yellow dwarf disease (Anonymous 1919) in Japan, as well as aster yellows disease (Kunkel 1926) in the United States, have been reported. These diseases were initially attributed to plant viruses because of their insect transmissibility and virus-like symptoms. However, Doi et al. (1967) discovered small pleomorphic bodies that resembled mycoplasmas (bacterial pathogens of humans and animals) in ultrathin sections of the phloem of plants affected by diseases such as mulberry dwarf, paulownia witches' broom, and aster yellows. The associated agents were named mycoplasma-like organisms (MLOs) because of their morphological similarity to mycoplasmas, as well as their sensitivity to tetracycline antibiotics (Ishiie et al. 1967). Discovery of these novel plant pathogens, MLOs, was confirmed in subsequent studies (Granados et al. 1968; Hull et al. 1969).

Molecular classification of phytoplasmas

More than four decades since their discovery, MLOs remain the most poorly characterized phytopathogens from every viewpoint, including biology and taxonomy, because they are difficult to culture in vitro. Moreover, since classification of MLOs was not possible, each MLO was named based on its natural host and symptoms. According to this nomenclature, as of the late 1980s, several hundred MLOs had been reported globally, and 63 MLOs were reported in Japan (Kishi 1987). In the early 1990s, the 16S rRNA gene sequences of MLOs were compared with each other, as well as with *Acholeplasma laidlawii*, *Spiroplasma citri*, and several mycoplasmas (Kuske and Kirkpatrick 1992; Namba et al. 1993b). These analyses revealed that MLOs were a monophyletic group within the class Mollicutes, but were more closely related to *Acholeplasma* spp. than to *Spiroplasma* spp. and animal mycoplasmas. In 1994, the name "phytoplasmas" was adopted by the Phytoplasma Working Team at the 10th Congress of the International Organization of Mycoplasmology to collectively denote MLOs. In 2004, phytoplasmas were proposed to be within a novel genus '*Candidatus* Phytoplasma' (IRPCM 2004). Approximately 40 phytoplasmal species have been reported worldwide (Hogenhout et al. 2008; Jung et al. 2002, 2003b, c; Sawayanagi et al. 1999). In Japan, approximately 70 strains of phytoplasmas (PSJ NIAS 2012) have been reclassified into nine species (Fig. 2).

Diagnosis of phytoplasma diseases

Phytoplasmas cause devastating damage to many plant species worldwide. For example, in 2001, a phytoplasma

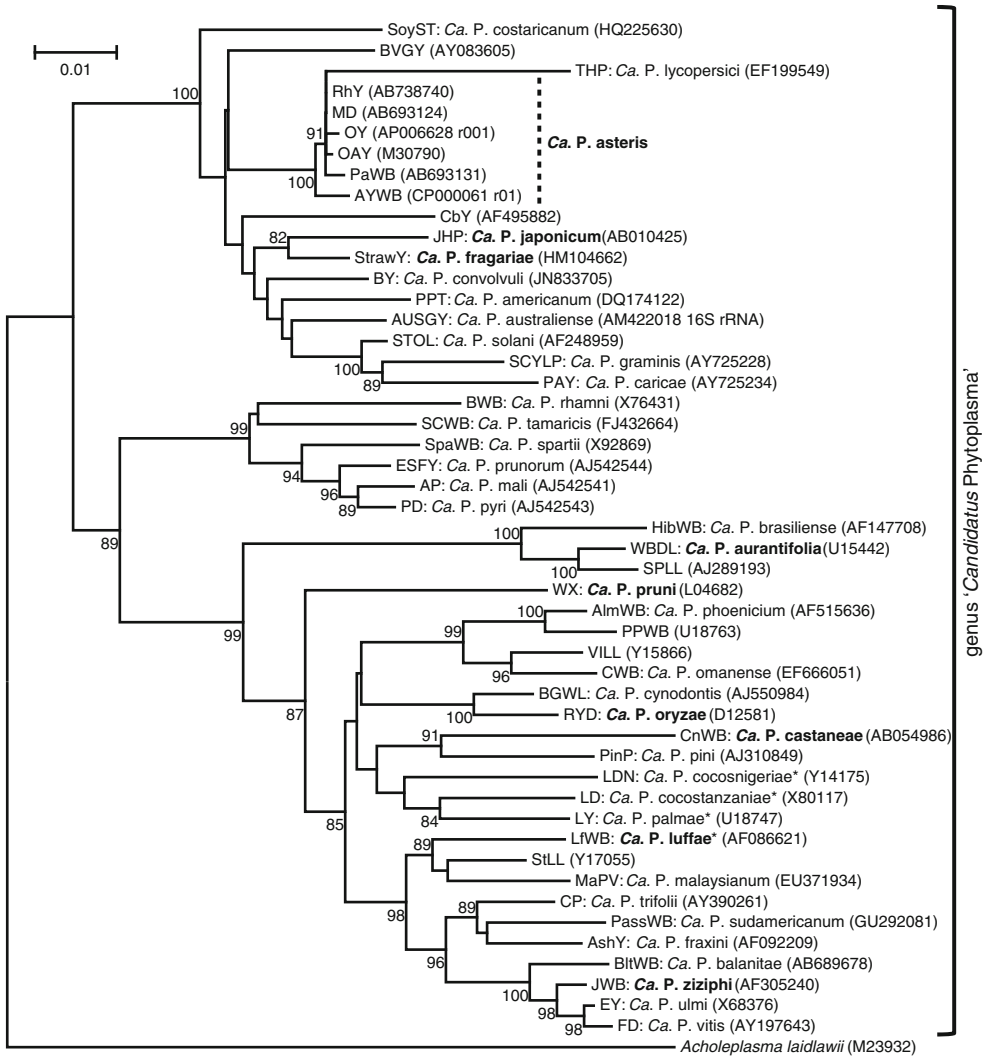

Fig. 2 Phylogenetic relationship of phytoplasmas. A phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei 1987) with 16S rRNA gene sequences from phytoplasmas and acholeplasma (outgroup). Numbers on the branches are the bootstrap values (only values >80 % are shown). GenBank accession numbers are in parentheses. *Asterisks* indicate the proposed provisional names (IRPCM 2004). *Scale bar* indicates the number of nucleotide substitutions per site. Species found in Japan are in *bold type*. *AlmWB* almond witches' broom, *AP* apple proliferation, *AshY* ash yellows, *AUSGY* Australian grapevine yellows, *AY-WB* aster yellows phytoplasma strain witches' broom, *BGWL* Bermuda grass white leaf, *BltWB* Balanites triflora witches' broom, *BVGY* Buckland Valley grapevine yellows, *BWB* buckthorn witches' broom, *BY* bindweed yellows, *Ca. P. Candidatus* Phytoplasma, *CbY* chinaberry yellows, *CnWB* chestnut witches' broom, *CP* clover proliferation, *CWB* cassia witches' broom, *ESFY* European stone fruit yellows, *EY* elm yellows, *FD* flavescence dorée of grapevine, *HibWB* hibiscus witches' broom, *JHP* Japanese hydrangea phyllody, *JWB* jujube witches' broom, *LD* coconut lethal yellowing, substrain Tanzanian lethal disease, *LDN* coconut lethal yellowing, substrain Nigerian Awka disease, *LY* coconut lethal yellowing, *LfWB* loofah witches' broom, *MaPV* Malaysian periwinkle virescence, *MD* mulberry dwarf, *OAY* oenothera aster yellows, *OY* onion yellows, *PassWB* passion fruit witches' broom, *PaWB* paulownia witches' broom, *PAY* papaya, *PD* pear decline, *PinP* pine phytoplasma, *PPT* potato purple top wilt, *PPWB* Caribbean pigeon pea witches' broom, *RhY* rhus yellows, *RYD* rice yellow dwarf, *SCWB* salt cedar witches' broom, *SCYLP* sugarcane yellow leaf syndrome, *SoyST* soybean stunt, *SpaWB* spartium witches' broom, *SPLL* sweet potato little leaf, *STOL* stolbur, *StrawY* strawberry yellows, *THP* 'Hoja de perejil' (parsley leaf) of tomato, *ViLL* vigna little leaf, *WBDL* witches' broom disease of lime, *WX* western X-disease

outbreak in apple trees caused losses of about €100 million in Italy and €25 million in Germany (Strauss 2009). Lethal yellowing of palm has killed millions of coconut palm trees in the Caribbean over the past 40 years (Brown et al. 2006). In Japan, phytoplasmas also cause serious economic losses to agricultural production.

'*Candidatus* Phytoplasma asteris' PaWB strain (PaWB) is the causal agent of paulownia witches' broom disease, an important paulownia tree disease. Once infected with PaWB, tree vigor is reduced, occasionally followed by dieback. Until 1960, more than 1 million PaWB-infected trees were found in Japan, excluding Hokkaido Prefecture

and the Tohoku region (Ito 1960), and the disease later spread to almost every plantation in the Tohoku region (Nakamura et al. 1998). Paulownia witches' broom disease also occurs in China (Hiruki 1999), where it has affected 880,000 ha of plantations (Yue et al. 2008). '*Candidatus* Phytoplasma oryzae' RYD strain (RYD) is the causal agent of rice yellow dwarf disease, a serious rice disease in many regions of Asia. Early infection with RYD results in a nearly 80 % yield loss. In the 1960s, rice yellow dwarf disease occurred in 10–50 % of rice growing areas of Japan and caused serious economic losses in many prefectures (Komori 1966; Sameshima 1967). In other cases, sweet potato witches' broom disease threatened the food supply in the Ryukyu Islands in the 1950s and 1960s (Nakamori and Maezato 1968; Shinkai 1964), and hydrangea phyllody disease remains a destructive disease in hydrangea cultivation (Kanehira et al. 1996; Sawayanagi et al. 1999; Takinami et al. 2013).

There is no effective way to control phytoplasma diseases; thus, early detection and removal of infected plants are critical to prevent the spread of disease. Until the early 1980s, phytoplasma diseases were diagnosed by transmission electron microscopic (TEM) observation because of their small size and the difficulty of culturing phytoplasmas in vitro. However, that approach requires expensive TEM equipment and time to prepare ultrathin sections of phloem tissue. In the 1980s, simple diagnostic techniques for phytoplasma diseases, such as direct fluorescence detection (DFD) (Namba et al. 1981) and DAPI staining (Hiruki and da Rocha 1986), were developed, both of which utilize florescent microscopy. The DFD method detects autofluorescence of necrotic phloem cells, and DAPI detects phytoplasmal DNA. Enzyme-linked immunosorbent assay (ELISA), the most prevalent method to diagnose viral diseases, was rarely used to detect phytoplasmal diseases in the 1980s, except for a few successful cases (Lin and Chen 1985), because it was difficult to prepare a phytoplasma-specific antibody at that time due to difficulties in purifying phytoplasmal cells.

Around 1990, advances in molecular biology enabled direct detection of phytoplasmal DNA by DNA–DNA hybridization (Kirkpatrick et al. 1987) and the polymerase chain reaction (PCR) (Deng and Hiruki 1991). PCR amplification of the 16S rRNA genes of phytoplasmas (Lee et al. 1993; Namba et al. 1993a; Schneider et al. 1995) has become the gold standard in the diagnosis of phytoplasmal diseases. PCR has also been applied to analyze phytoplasmal localization and dynamics in planta (Nakashima and Hayashi 1995b; Sahashi et al. 1995; Wei et al. 2004b). Recently, loop-mediated isothermal amplification (LAMP) (Notomi et al. 2000) has also been used to detect several phytoplasmas and is expected to become a rapid and reliable field-diagnostic system for phytoplasma diseases (Sugawara et al. 2012; Tomlinson et al. 2010). LAMP is more sensitive and rapid than PCR amplification and does not require DNA purification or special equipment such as a thermal cycler. The first LAMP-based detection kit for phytoplasmas has been commercially available since 2011 in Japan (http://nippongene-analysis.com/phytoplasma-fs.htm). Application of the kit for on-site diagnosis will aid in the early control of phytoplasmal diseases.

Reductive evolution of the genomes

Biological and molecular characteristics of phytoplasmas are still quite unclear because of the difficulty in culturing them in vitro. However, in the late 1990s, several genome projects were initiated globally to explore the genomic features of phytoplasmas. At the same time, several mild-pathogenicity mutants (Shiomi et al. 1998; Uehara et al. 1999) and a non-insect-transmissible mutant (Oshima et al. 2001b) were identified in Japan. Among these mutant strains, the '*Ca.* P. asteris' OY-M strain (OY-M) was thought to be suitable for whole genome analysis because the chromosome size of OY-M (ca. 870 kbp) was much smaller than that of the '*Ca.* P. asteris' OY-W strain (OY-W; ca. 1,000 kbp) (Oshima et al. 2001b). In parallel with the OY-M genome project, the draft genomic sequences of OY-W were obtained (Oshima et al. 2002; Miyata et al. 2002b), revealing phytoplasma codon usage (Miyata et al. 2002a), catalytic activity assays of phytoplasmal proteins (Miyata et al. 2003), and the presence of two rRNA operons (Jung et al. 2003a). In 2004, the complete genomic sequence of OY-M was determined (Oshima et al. 2004). To date, the complete genomic sequences of three other phytoplasmas have been reported (Bai et al. 2006; Tran-Nguyen et al. 2008; Kube et al. 2008).

Although phytoplasma genomes contain genes for basic cellular functions such as DNA replication, transcription, translation, and protein translocation, they lack genes for amino acid biosynthesis, fatty acid biosynthesis, the tricarboxylic acid cycle, and oxidative phosphorylation, just as in the mycoplasma genome (Razin et al. 1998). Phytoplasma genomes, however, encode even fewer metabolically functional proteins than do mycoplasma genomes, which were previously thought to have the minimum possible gene set (Mushegian and Koonin 1996). For example, phytoplasma genomes lack the pentose phosphate pathway genes and genes encoding F_1F_o-type ATP synthase. Therefore, ATP synthesis in phytoplasmas is strongly dependent on glycolysis instead of ATP synthase (Oshima et al. 2007). Interestingly, phytoplasmas harbor multiple copies of transporter-related genes not found in mycoplasmas. These genomic features suggest that phytoplasmas are highly dependent on metabolic compounds

Table 1 Summary of insect vectors of phytoplasma diseases in Japan

Disease name	Host plant	Causal phytoplasma species	Insect vector	References
Anemone witches' broom	*Anemone coronaria*	*Ca.* P. asteris	*Macrosteles striifrons*	Namba (1996)
Buplever yellows	*Bupleurum falcatum*	*Ca.* P. asteris	*Macrosteles striifrons*	Namba (1996)
Carrot yellows	*Daucus carota*	*Ca.* P. pruni	*Ophiola flavopicta*	Namba (1996)
Celery yellows	*Apium graveolens*	*Ca.* P. pruni	*Ophiola flavopicta*	Namba (1996)
China aster yellows	*Callistephus chinensis*	*Ca.* P. pruni	*Ophiola flavopicta*	Namba (1996)
Cineraria witches' broom	*Senecio* × *hybridus*	*Ca.* P. asteris	*Macrosteles striifrons*	Namba (1996)
Cosmos yellows	*Cosmos bipinnatus*	*Ca.* P. asteris	*Macrosteles striifrons*	Wakibe et al. (1995)
Cryptotaenia japonica witches' broom	*Cryptotaenia japonica*	*Ca.* P. asteris	*Macrosteles striifrons*	Namba (1996)
			Hishimonus sellatus	Nishimura et al. (1998)
			Hishimonoides sellatiformis	Nishimura et al. (2004)
Garland chrysanthemum witches' broom	*Chrysanthemum coronarium* var. *spatiosum*	*Ca.* P. asteris	*Macrosteles striifrons*	Shiomi and Sugiura (1983)
Gentian witches' broom	*Gentiana scabra* var. *buergeri*	*Ca.* P. pruni	*Ophiola flavopicta*	Namba (1996)
		Ca. P. asteris	*Macrosteles striifrons*	Tanaka et al. (2006)
Geranium witches' broom	*Pelargonium* × *hortorum*	*Ca.* P. pruni	*Ophiola flavopicta*	Namba (1996)
Glaucidium witches' broom	*Glaucidium palmatum*	*Ca.* P. pruni	*Ophiola flavopicta*	Tanaka et al. (1998)
Hovenia witches' broom	*Hovenia tomentella*	*Ca.* P. asteris	*Hishimonus sellatus*	Kusunoki et al. (2002)
Iceland poppy yellows	*Papaver nudicaule*	*Ca.* P. asteris	*Macrosteles striifrons*	Namba (1996)
Jujube witches' broom	*Zizyphus jujuba*	*Ca.* P. ziziphi	*Hishimonus sellatus*	Kusunoki et al. (2002)
Legume witches' broom	(plants in the family Fabaceae)	*Ca.* P. aurantifolia	*Orosius orientalis*	Namba (1996)
Marguerite yellows	*Argyranthemum frutescens*	*Ca.* P. asteris	*Macrosteles striifrons*	Namba (1996)
Mulberry dwarf	*Morus* spp.	*Ca.* P. asteris	*Hishimonus sellatus*	Namba (1996)
			Hishimonoides sellatiformis	Namba (1996)
			Tautoneura mori	Jiang et al. (2005)
Onion yellows	*Allium cepa*	*Ca.* P. asteris	*Macrosteles striifrons*	Namba (1996)
			Hishimonus sellatus	Nishimura et al. (1998)
			Hishimonoides sellatiformis	Nishimura et al. (2004)
Potato witches' broom	*Solanum tuberosum*	*Ca.* P. pruni	*Ophiola flavopicta*	Namba (1996)
Rhus yellows	*Rhus javanica*	*Ca.* P. asteris	*Hishimonus sellatus*	Tanaka et al. (2000)
Rice yellow dwarf	*Oryza sativa*	*Ca.* P. oryzae	*Nephotettix cincticeps*	Namba (1996)
			Nephotettix nigropictus	Namba (1996)
			Nephotettix virescens	Namba (1996)
Rocket larkspur witches' broom	*Consolida ambigua*	*Ca.* P. asteris	*Macrosteles striifrons*	Tanaka et al. (2007)
Statice witches' broom	*Limonium* spp.	*Ca.* P. asteris	*Macrosteles striifrons*	Shiomi et al. (1999)
Strawberry witches' broom	*Fragaria* × *ananassa*	*Ca.* P. asteris	*Macrosteles striifrons*	Namba (1996)
Sweet potato witches' broom	*Ipomoea batatas*	*Ca.* P. aurantifolia	*Orosius ryukyuensis*	Namba (1996)
Tomato yellows	*Lycopersicon esculentum*	*Ca.* P. pruni	*Ophiola flavopicta*	Namba (1996)
		Ca. P. asteris	*Macrosteles striifrons*	Kato et al. (1988)
Tsuwabuki witches' broom	*Farfugium japonicum*	*Ca.* P. pruni	*Ophiola flavopicta*	Namba (1996)
Udo dwarf	*Aralia cordata*	*Ca.* P. pruni	*Ophiola flavopicta*	Namba (1996)
Water dropwort yellows	*Oenanthe javanica*	*Ca.* P. asteris	*Macrosteles striifrons*	Shiomi and Sugiura (1983)

Table 1 continued

Disease name	Host plant	Causal phytoplasma species	Insect vector	References
Welsh onion yellows	*Allium fistulosum*	*Ca*. P. asteris	*Macrosteles striifrons*	Shiomi et al. (1996)
White lace flower yellows	*Ammi majus*	*Ca*. P. asteris	*Macrosteles striifrons*	Namba (1996)
Chestnut yellows	*Castanea* spp.	*Ca*. P. castaneae	Unknown	–
Elaeocarpus yellows	*Elaeocarpus sylvestris* var. *ellipticus*	*Ca*. P. luffae	Unknown	–
Hydrangea phyllody	*Hydrangea* spp.	*Ca*. P. asteris	Unknown	–
		Ca. P. japonicum	Unknown	–
Paulownia witches' broom	*Paulownia tomentosa*	*Ca*. P. asteris	Unknown	–

from their hosts (Oshima et al. 2013). Phytoplasma genomes contain the *sodA* gene, which encodes superoxide dismutase that can inactivate reactive oxygen species (ROS) (Miura et al. 2012). Plants deploy a broad range of defenses during infection by various pathogens. The oxidative burst, which produces ROS, is one of the earliest events in the plant defense response. Since the genomes of mycoplasmas do not contain this gene, the presence of sodA may defend phytoplasmas against the unique threat of ROS released by the plant cell. Phytoplasma genomes also contain clusters of repeated gene sequences called potential mobile units (PMUs) (Bai et al. 2006), which consist of similar genes organized in a conserved order (Arashida et al. 2008a; Jomantiene and Davis 2006). A PMU in '*Ca*. P asteris' AY-WB strain (AY-WB) exists as both linear chromosomal and circular extrachromosomal elements (Toruño et al. 2010), suggesting the ability to transpose within the genome.

Characteristic features of the plasmids

In general, a phytoplasma genome consists of one chromosome and several small plasmids (Firrao et al. 2007). Phytoplasmal plasmids were cloned in the late 1980s (Davis et al. 1988; Nakashima et al. 1991) and have often been used as targets of DNA hybridization to compare phytoplasma strains (Nakashima and Hayashi 1995a). Complete nucleotide sequence analyses of the plasmids revealed that they may be products of DNA recombination between the phytoplasmal plasmids (Nishigawa et al. 2002a) and that each plasmid encodes a replication initiation protein (Rep) involved in rolling-circle replication, as well as several other unknown proteins (Nakashima and Hayashi 1997; Kuboyama et al. 1998; Nishigawa et al. 2003). Interestingly, the phytoplasmal Reps are similar to the Reps encoded by bacterial plasmids and to geminivirus (Nakashima and Hayashi 1997; Nishigawa et al. 2001) and circovirus Reps (Oshima et al. 2001a), suggestive of evolutionary relationships between phytoplasmas and viruses.

The functions of other genes in the phytoplasmal plasmids remain unknown. However, some genes are apparently related to adaptation to the insect host. For example, '*Ca.* P. asteris' OY-NIM strain (OY-NIM), a non-insect-transmissible derivative line of OY-M, lacked the ability to express the ORF3 gene encoded in plasmids (Nishigawa et al. 2002b; Ishii et al. 2009a). Sequence analysis of the plasmids over 10 years revealed that one of the plasmids was gradually lost from OY-NIM and finally disappeared during the maintenance of OY-NIM in plant tissue culture (Ishii et al. 2009b). These results suggested that the plasmid is not essential for phytoplasmal viability in a plant host, but may be a key element for adaption to an insect host.

Molecular mechanisms of insect transmissibility

Phytoplasmas are transmitted by specific insect species in a persistent manner. It is important to understand the molecular mechanisms underlying these strict pathogen–insect specificities in order to control phytoplasmal diseases. Many insect vectors involved in phytoplasma diseases have been identified in Japan (Table 1). Building on knowledge about the insect vectors, recent studies on phytoplasmal localization in nonvector insects revealed that the phytoplasma–insect specificity is determined by the ability to pass through several physical barriers, including the midgut and salivary gland (Nakajima et al. 2002, 2009).

Since phytoplasmas are endocellular parasites that lack a cell wall, their membrane proteins and secreted proteins function directly in the host cell. In many phytoplasmas, a subset of membrane proteins (usually referred to as immunodominant membrane proteins) accounts for a major portion of the total cellular membrane proteins (Kakizawa et al. 2006b). Immunodominant membrane proteins were classified into three types: immunodominant membrane protein (Imp) (Kakizawa et al. 2009), antigenic membrane protein (Amp) (Kakizawa et al. 2004), and immunodominant membrane protein A (IdpA) (Neriya et al. 2011),

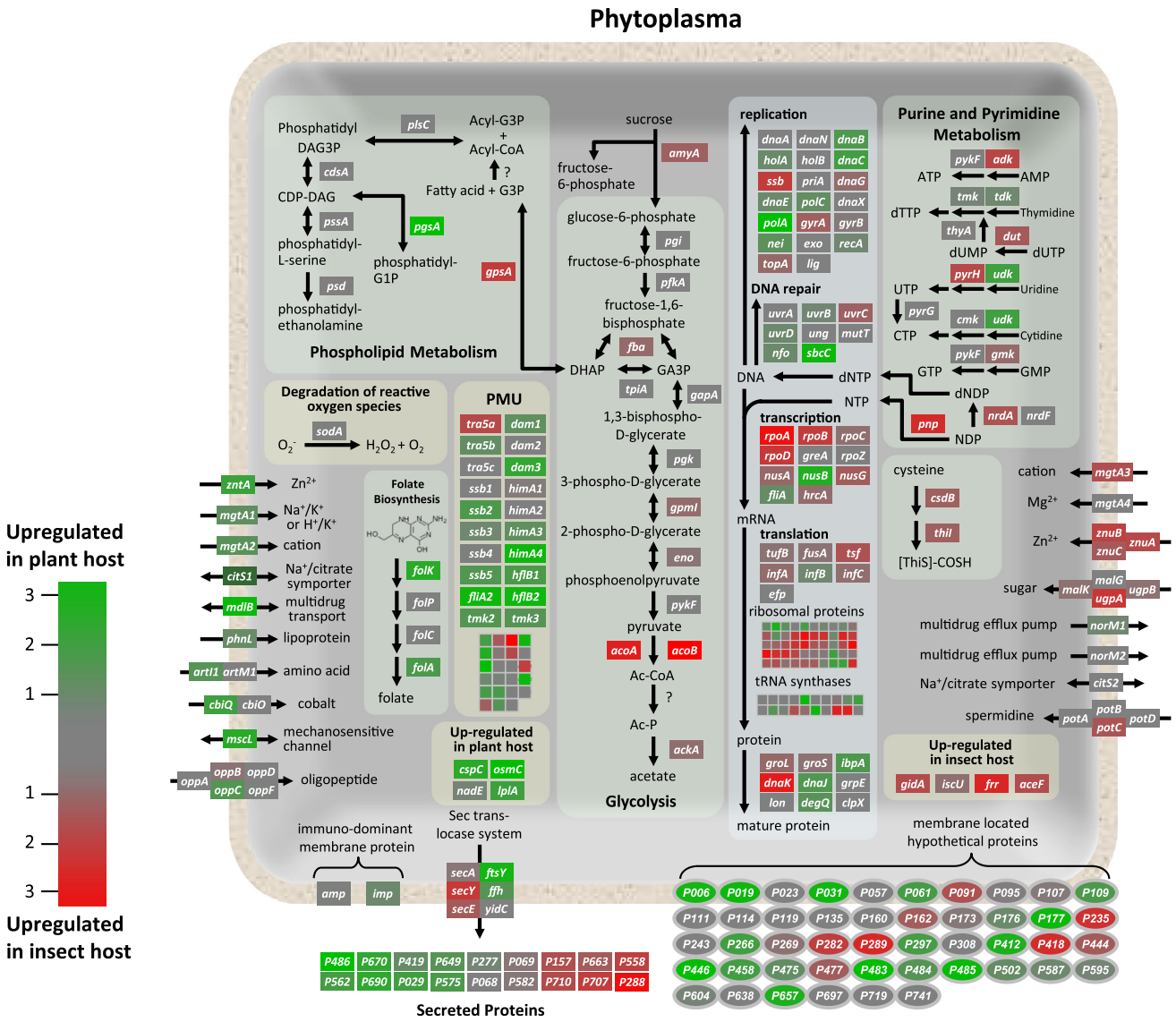

Fig. 3 Global patterns of gene regulation in phytoplasma associated with host switching between plant and insect (modified from Oshima et al. 2011). Genes upregulated in the plant host are in green; those upregulated in the insect host are in *red*

which are not orthologues. In both OY-W and OY-M, Amp was a major surface membrane protein (Kakizawa et al. 2009). Cloning of *amp* genes from several phytoplasma strains showed that Amp proteins are under positive selection, and positively selected amino acids can be found in the central hydrophilic domain (Kakizawa et al. 2006a). In addition, the Amp protein forms a complex with insect microfilaments (Suzuki et al. 2006). Interestingly, the formation of Amp–microfilament complexes was correlated with the phytoplasma-transmitting capability of leafhoppers, suggesting that the interaction between Amp and insect microfilaments may play a major role in determining the transmissibility of phytoplasmas (Suzuki et al. 2006). The interaction between Amp and actin (a component of microfilament), as well as the ATP synthase β subunit of insect vectors, has also been observed for the CYP strain of '*Ca*. P. asteris' (Galetto et al. 2011).

Moreover, a phytoplasmal microarray analysis of OY-M revealed that expression of approximately one-third of the genes is affected during host switching between plant and insect, suggesting that the phytoplasma alters its gene expression in response to its host (Fig. 3) and may use transporters, membrane proteins, secreted proteins, and metabolic enzymes in a host-specific manner (Oshima et al. 2011).

Virulence factors involved in unique symptoms

Phytoplasmas cause dramatic changes in plant development (Arashida et al. 2008b); however, the molecular

mechanisms underlying their pathogenicity remain unclear. The phytoplasma genome lacks homologs of the type III secretion system, which are essential for the virulence of most phytopathogenic bacteria (Abramovitch et al. 2006), and for toxins produced by *Pseudomonas* spp., cell wall maceration enzymes of *Pectobacterium* spp., and the type IV secretion system of *Rhizobium* (*Agrobacterium*) spp. (Oshima et al. 2004). On the other hand, phytoplasmas have no cell walls and reside within host cells, and their secreted proteins are believed to directly function in the host plant or insect cells. The genes encoding SecA, SecY, and SecE, required for protein translocation in *Escherichia coli* (Economou 1999), were identified in strain OY-M of '*Ca*. P. asteris' (Kakizawa et al. 2001, 2004) and in three other phytoplasma genomes (Bai et al. 2006; Kube et al. 2008; Tran-Nguyen et al. 2008). In addition, SecA protein was expressed in plants infected with five respective phytoplasma species (Wei et al. 2004a). These results suggest that the Sec system is widely conserved among phytoplasmas.

Homologs of the virulence genes of other phytopathogenic bacteria were not identified in the phytoplasma genomes. Therefore, phytoplasmal proteins secreted via the Sec system are candidate virulence factors that induce morphological changes. In 2009, a secreted protein (TENGU) was identified from OY-M as the first phytoplasmal virulence factor (Hoshi et al. 2009). TENGU induces witches' broom and dwarfism, which are typical of phytoplasma infection in *Nicotiana benthamiana* and *Arabidopsis thaliana*. TENGU homologs from several phytoplasma strains induce similar symptoms (Sugawara et al. 2013). Microarray analysis of TENGU-transgenic *Arabidopsis* plants revealed that TENGU inhibits auxin-related pathways, thereby affecting plant development (Fig. 4) (Hoshi et al. 2009). More recently, TENGU was found to be processed in planta into a small functional peptide, similar to the proteolytic processing of plant endogenous peptide signals (Sugawara et al. 2013). As for phyllody symptoms, a previous study has shown the involvement of the floral homeotic MADS-domain transcription factors of the floral ABCE model (floral quartet model) (Himeno et al. 2011). Recently, PHYL1 and SAP54 were found to be homologous secreted proteins that induce phyllody in floral organs of *Arabidopsis thaliana* (MacLean et al. 2011; Maejima et al. 2014). PHYL1 actually interacts with and degrades these MADS-domain transcription factors (Maejima et al. 2014). *PHYL1* gene was genetically and functionally conserved among other phytoplasma strains and species. Therefore, *PHYL1* and its homologs were designated as members of the phyllody-inducing gene family "phyllogen." SAP11, a secreted protein from AY-WB, contains eukaryotic nuclear localization signals and localizes in plant cell nuclei (Bai et al. 2009). SAP11 downregulates JA synthesis and increases

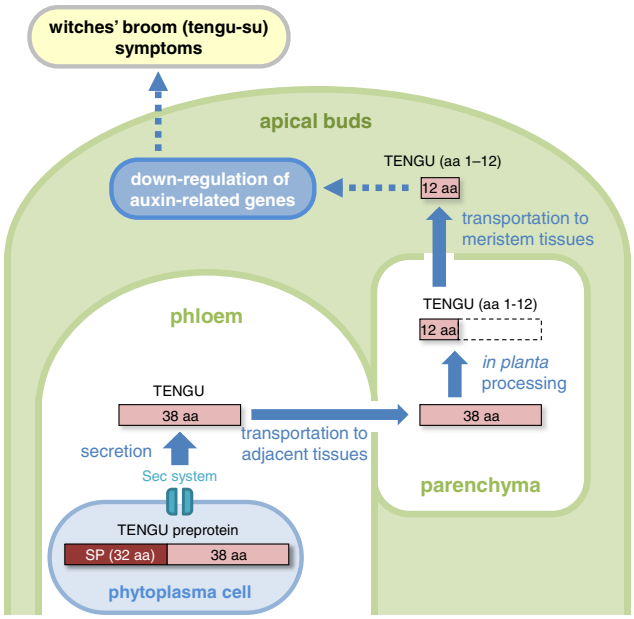

Fig. 4 Hypothetical mechanism of witches' broom (tengu-su) symptoms induced by a phytoplasmal virulence factor, TENGU. TENGU, a small peptide secreted from the phytoplasma, is translated as a 70-amino acid preprotein with a signal peptide (SP) of 32 amino acids (aa) at its N terminus. The C-terminal 38 aa of TENGU preprotein are secreted into plant phloem via the Sec system, a bacterial conserved protein translocation system on the cellular membrane, where the N-terminal signal peptide is cleaved. TENGU is transported from phloem into parenchyma and apical buds. During the transportation, TENGU is proteolytically processed into the active form consisting of N-terminus 12 aa. The active form of TENGU inhibits auxin-related pathways, resulting in induction of witches' broom (tengu-su) symptoms

the fecundity of insect vectors (Sugio et al. 2011). In addition to these secreted proteins, the consumption of plant metabolites by phytoplasmas may be associated with virulence. The duplicated glycolytic genes in OY-W are likely responsible for the severe symptoms (Oshima et al. 2007). Moreover, in the most recent study, activation of the host anthocyanin biosynthesis pathway in response to phytoplasma infection is responsible for purple top symptoms and is associated with a reduction of leaf cell death (Himeno et al. 2014). Through all these studies, the molecular mechanisms of phytoplasma symptoms are becoming clear. On the other hand, the biological significance of the unique symptoms, which is also an interesting topic, remains largely unknown, and awaits further study.

Concluding remarks

Although phytoplasmas remain the most poorly characterized phytopathogens, recent studies have identified virulence factors that induce typical phytoplasma disease

symptoms and have characterized the unique reductive evolution of the genome. Phytoplasma-related diseases are expected to increase because the warming global climate is advantageous to the cold-sensitive vectors of the phytoplasmas. Therefore, pest control and detection of phytoplasmas are important. Further analysis of phytoplasmas at the molecular level will increase our understanding of these economically important and biologically fascinating microorganisms.

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